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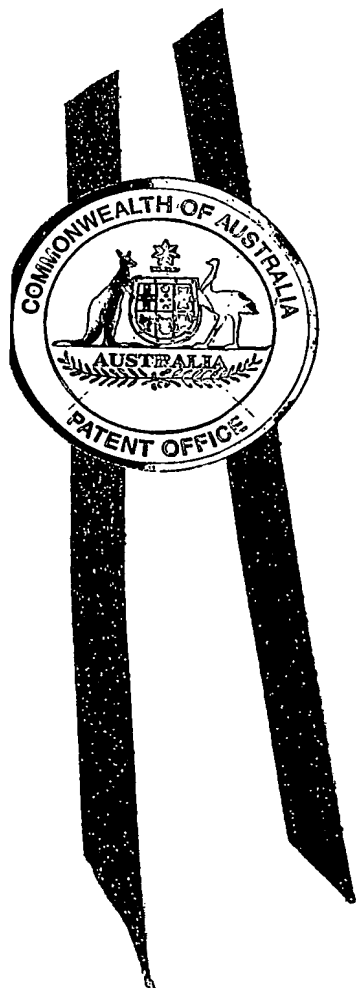
I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003903850 for a patent by BIOTRON LIMITED as filed on 25 July 2003.

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WITNESS my hand this  
Seventh day of July 2004

A handwritten signature in cursive script that reads "J. Billingsley".

JULIE BILLINGSLEY  
TEAM LEADER EXAMINATION  
SUPPORT AND SALES



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DOCUMENT**  
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**AUSTRALIA**

**PATENTS ACT 1990**

**PROVISIONAL SPECIFICATION**

***FOR THE INVENTION ENTITLED:-***

**"ANTI-CORONAVIRUS COMPOUNDS AND METHODS"**

The invention is described in the following statement:-

## ANTI-CORONAVIRUS COMPOUNDS AND METHODS

### FIELD OF THE INVENTION

The present invention relates to compounds and methods for retarding, reducing or otherwise inhibiting growth, or infection by, coronaviruses. In particular the invention relates to compositions and methods for treating Severe Acute Respiratory Syndrome (SARS), the common cold and other conditions caused by exposure to or infection with a coronavirus.

### BACKGROUND OF THE INVENTION

Coronaviruses (Order *Nidovirales*, family *Coronaviridae*, Genus *Coronavirus*) are enveloped positive-stranded RNA viruses, that bud from the endoplasmic reticulum-Golgi intermediate compartment or the *cis*-Golgi network (Fischer, Stegen et al. 1998; Maeda, Maeda et al. 1999; Corse and Machamer 2000; Maeda, Repass et al. 2001; Kuo and Masters 2003)

Coronaviruses infect humans and animals and it is thought that there could be a coronavirus that infects every animal. The two human coronaviruses, 229E and OC43, are known to be the major causes of the common cold and can occasionally cause pneumonia in older adults, neonates, or immunocompromised patients (Peiris, Lai et al. 2003). Animal coronaviruses can cause respiratory, gastrointestinal, neurological, or hepatic diseases in their host (Peiris, Lai et al. 2003). Several animal coronavirus are significant veterinary pathogens (Rota, Oberste et al. 2003).

Severe acute respiratory syndrome (SARS) is caused by a newly identified virus. SARS is a respiratory illness that has recently been reported in Asia, North America, and Europe (Peiris, Lai et al. 2003). The causative agent of SARS was identified as a coronavirus. (Drosten, Gunther et al. 2003; Ksiazek, Erdman et al. 2003; Peiris, Lai et al. 2003). The World Health Organization reports that the cumulative number of reported probable cases of SARS from 1 November 2002 to the 11<sup>th</sup> July 2003 is 8,437 with 813 deaths, nearly a 10% death rate. It is believed that SARS will not be eradicated, but will cause seasonal epidemics like the cold or influenza viruses (Vogel 2003)

## SUMMARY OF THE INVENTION

It was surprisingly found that cinnamoylguanidine and its analogues or derivatives, are able to impact negatively on the growth of the SARS virus and other coronaviruses. With relevance to other coronaviruses, and without being  
5 bound by any particular theory or mechanism of action, the effect of the compounds appears to be exerted via the inhibition, or other negative effect, of the E protein of the these viruses which were shown in the present studies to act as ion channels. As similar E proteins are present in other coronaviruses, the compositions and methods of the present invention would have utility in the  
10 inhibition and/or treatment of infections by other coronaviruses.

Thus, according to a first aspect of the present invention provides a compound selected from the group consisting of cinnamoylguanidines and naphthoylguanidines, or analogues and derivatives thereof, and amiloride analogues or derivatives thereof, having anti-coronavirus activity.

15 According to a second aspect, the present invention provides a compound selected from the group consisting of cinnamoylguanidines and naphthoylguanidines, or analogues and derivatives thereof, and amiloride analogues or derivatives thereof, capable of retarding, reducing or otherwise inhibiting growth of a coronavirus.

20 According to a third aspect, the present invention provides a method of retarding, reducing or otherwise inhibiting growth of a coronavirus comprising contacting or exposing said virus to a compound selected from the group consisting of cinnamoylguanidines and naphthoylguanidines, or analogues and derivatives thereof, and amiloride analogues or derivative thereof.

25 According to a fourth aspect, the present invention provides a method of prophylactic or therapeutic treatment of a coronavirus infection comprising the administration to a subject requiring such treatment of a compound selected from the group consisting of cinnamoylguanidines and naphthoylguanidines, or analogues and derivatives thereof, and amiloride analogues or derivative  
30 thereof

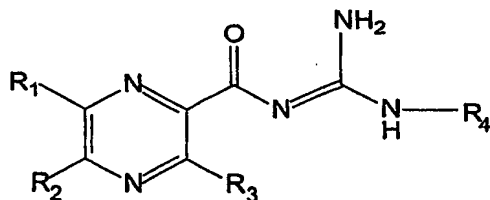
Preferably the coronavirus to be inhibited or infection treated is that of the SARS virus.

Even more preferably, the coronaviruses to be inhibited or infection treated are those of the 229E or OC43 viruses.

Other coronaviruses which can be inhibited or infections treated are those listed in Table 1.

- 5 Examples of suitable compounds that can be used in the compositions and methods of the present invention are listed below.

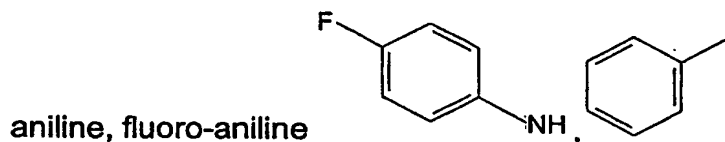
Amiloride analogues or derivatives comprising the structure:



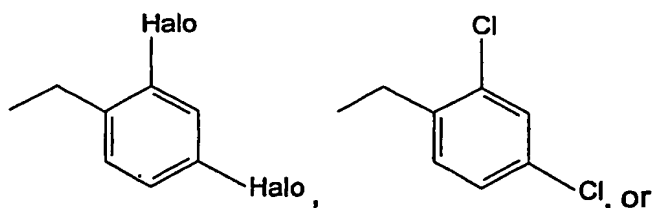
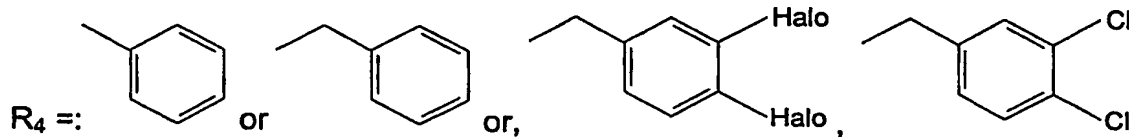
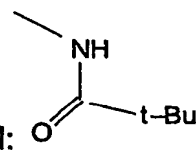
wherein the substituents at the R positions may or maynot be the same, and

- 10  $R_1$  = halo, aryl, substituted aryl, phenyl, or substituted phenyl;

$R_2$  = amine, aryl, substituted aryl, phenyl, substituted phenyl, hexamethylene, PrS, N-methyl-N-isobutyl, N-ethyl -N-isopropyl, benzyl ; N-methyl-N-guanidinocarbonyl-methyl, N,N-dimethyl, N,N-diethyl, tert-butylamino, halo-

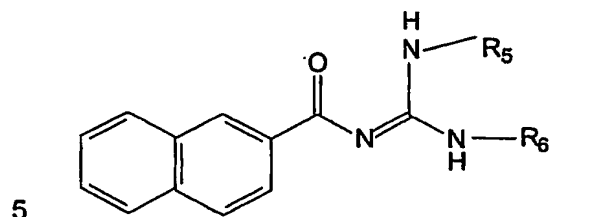


- 15  $R_3$  = hydroxy, alkyloxy, methoxy, N,3-dimethylbutanamyl:

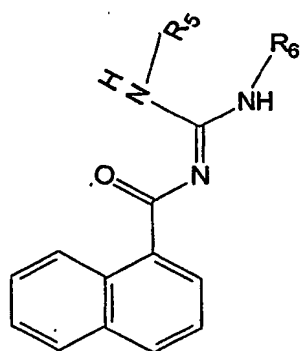


- 20 , — BOD<sup>IPY-FL</sup>,

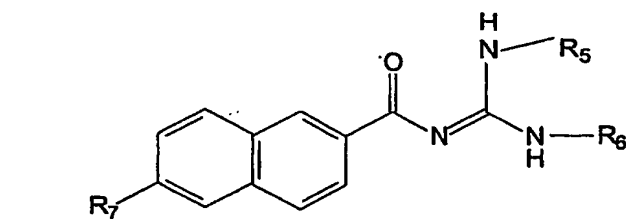
The following antiviral compounds comprising a guanidyl moiety are also encompassed within the scope of the present invention:



or



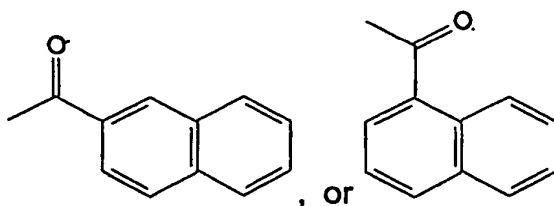
or



wherein the substituents at the R positions may or may not be the same, and

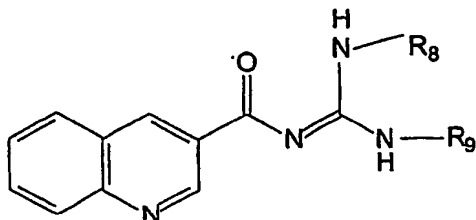
$R_5 = \text{H, aryl, substituted aryl, phenyl, or substituted phenyl};$

$R_6 = \text{H, aryl, substituted aryl, phenyl, substituted phenyl, naphthoyl,}$



$R_7 = \text{alkyloxy, or methoxy};$

15 or, the structure

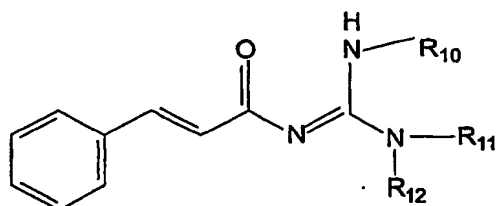


wherein the substituents at the R positions may or may not be the same, and

$R_8$  = aliphatic or aromatic substituents;

$R_9$  = aliphatic or aromatic substituents;

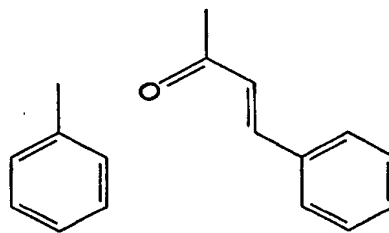
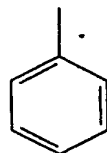
5 or, the structure



wherein the substituents at the R positions may or may not be the same, and

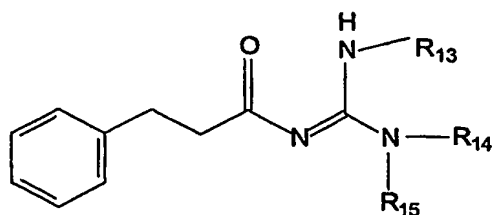
$R_{10}$  = H, aryl, phenyl, or cinnamoyl;

$R_{11}$  = H, alkyl, aryl, phenyl, cinnamoyl; or



10  $R_{12}$  = H, alkyl, aryl, phenyl, cinnamoyl, or

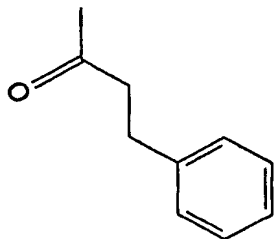
or, the structure



wherein the substituents at the R positions may or may not be the same, and

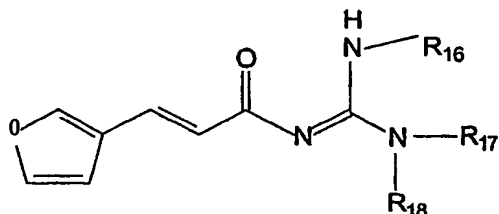
$R_{13}$  = H; alkyl, or phenyl

15  $R_{14}$  = H, alkyl, phenyl, or substituted phenyl, -



$R_{15} = H$ , or

or, the structure



wherein the substituents at the R positions may or may not be the same, and

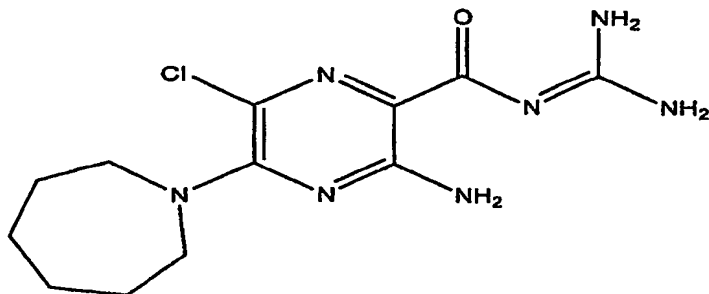
5  $R_{16} = H$ , alkyl, substituted alkyl, phenyl, or substituted phenyl;

$R_{17} = H$ , alkyl, substituted alkyl, phenyl, or substituted phenyl;

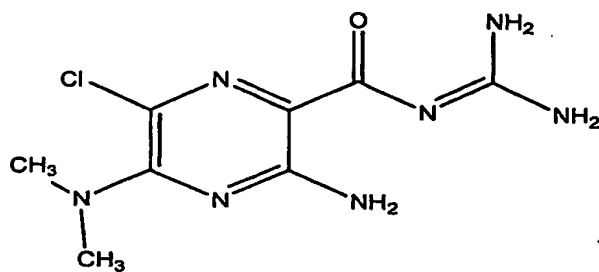
$R_{18} = H$ , alkyl, substituted alkyl, phenyl, or substituted phenyl;

The compounds of the invention include the following:

5-(N,N-hexamethylene)amiloride

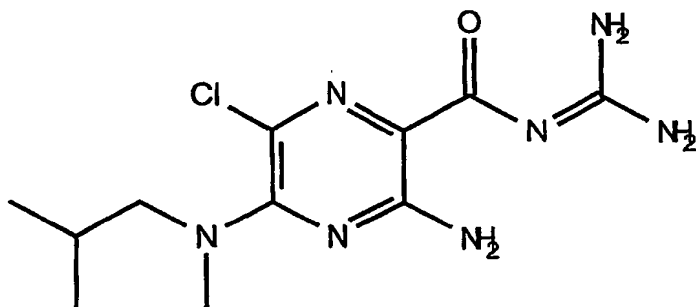


10 5-(N,N-Dimethyl)amiloride hydrochloride

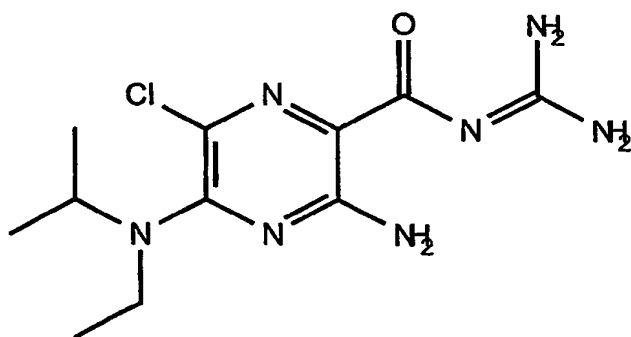


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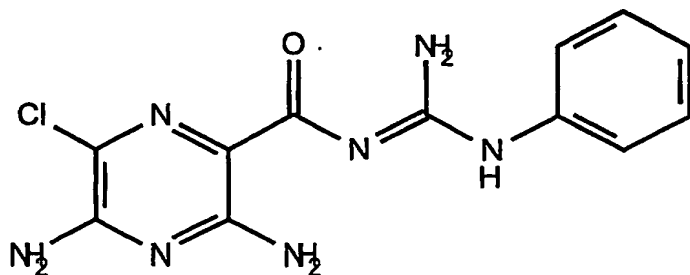
5-(N-methyl-N-isobutyl)amiloride comprising the structure



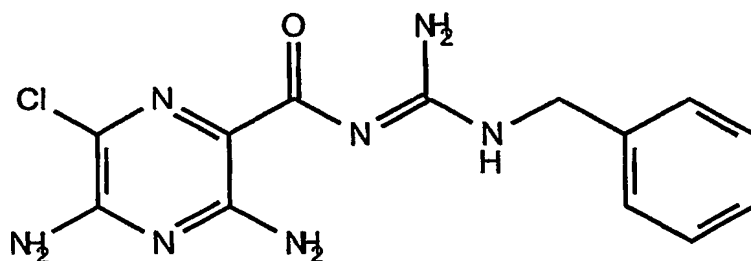
5 5-(N-ethyl-N-isopropyl)amiloride (herein referred to as EIPA), comprising the structure



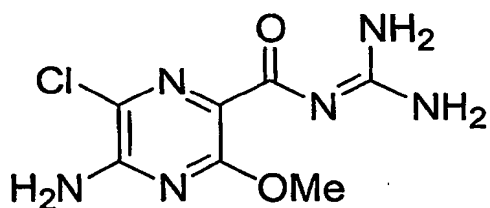
10 N-(3,5-Diamino-6-chloro-pyrazine-2-carbonyl)-N'-phenyl-guanidine, comprising the structure



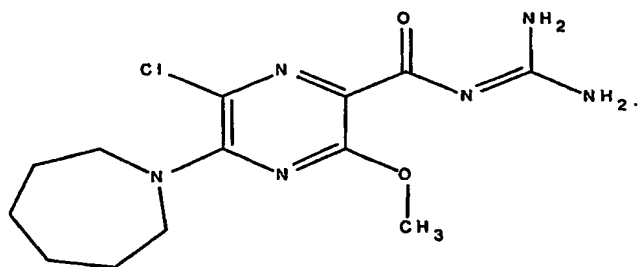
N-Benzyl -N'-(3,5-diamino-6-chloro-pyrazine-2-carbonyl)-guanidine, comprising the structure



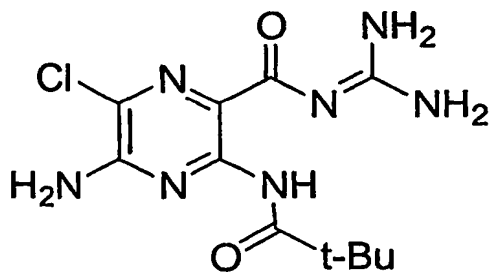
5 3-methoxy amiloride comprising the structure



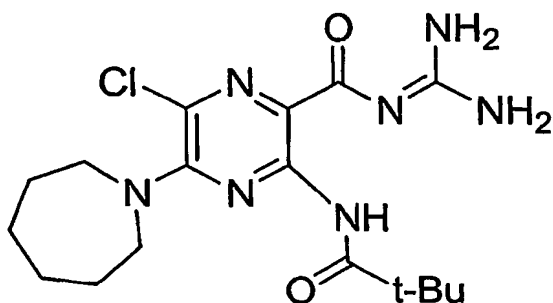
3-methoxy-5-(N,N-Hexamethylene)-amiloride comprising the structure



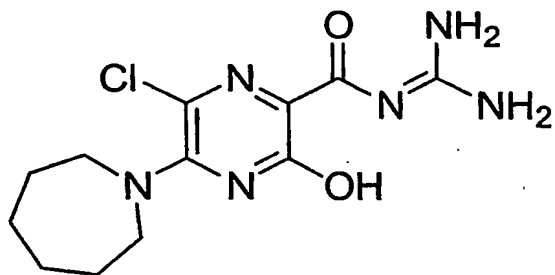
3-(N-2,2 -dimethyl propanal)amiloride comprising the structure



3-(N-2,2 -dimethyl propanal)-5-N-hexamethylene amiloride comprising the structure

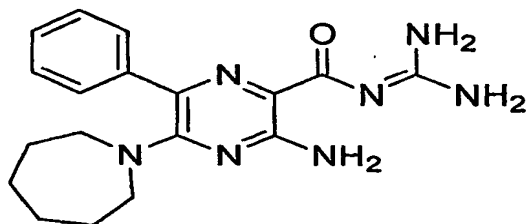


3-hydroxy-5-hexamethyleneimino-amiloride comprising the structure



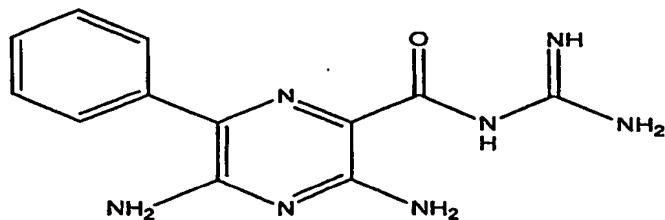
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Hexamethyleneimino-6-phenyl-2-pyrazinecarboxamide comprising the structure

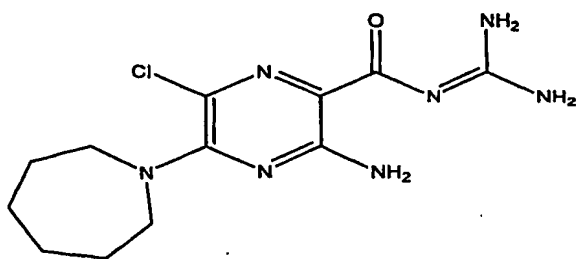


N-amidino-3,5-diamino-6-phenyl-2-pyrazinecarboxamide comprising the structure

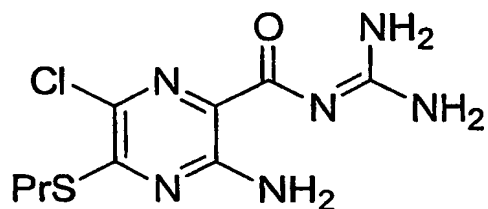
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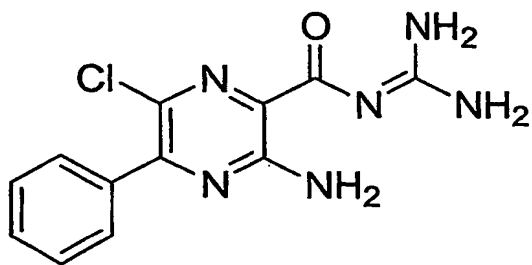
5-(N,N-hexamethylene)amiloride comprising the structure



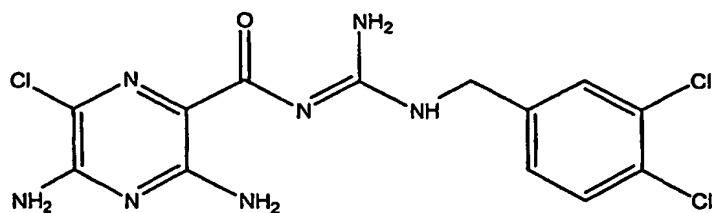
5-propyl-sulfide amiloride comprising the structure



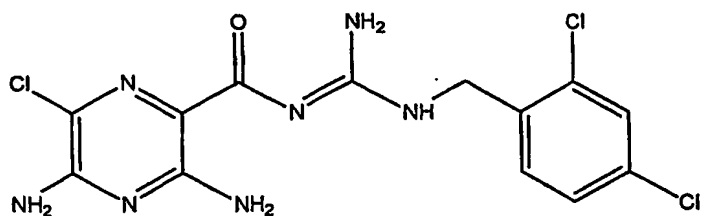
5 N-amidino-3-amino-5-phenyl-6-chloro-2-pyrazinecarboxamide comprising the structure



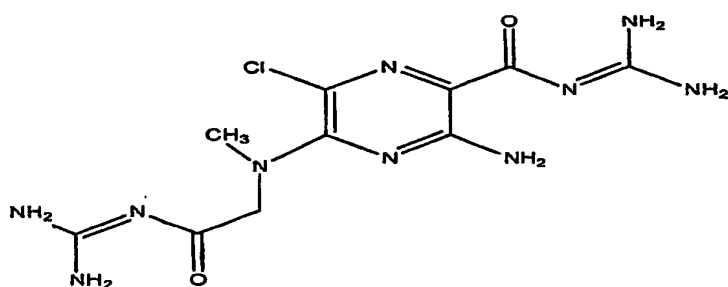
3,4-Dichlorobenzamil comprising the structure



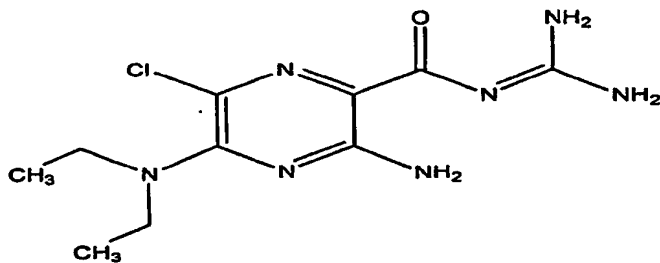
2'4 DichloroBenzamil HCl comprising the structure



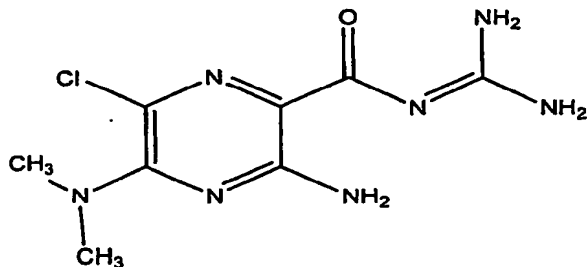
5-(N-methyl-N-guanidinocarbonyl-methyl)amiloride comprising the structure



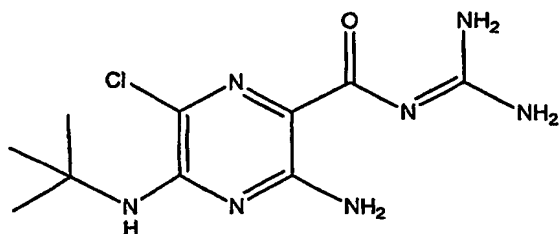
5 5-(N,N-Diethyl)amiloride hydrochloride comprising the structure



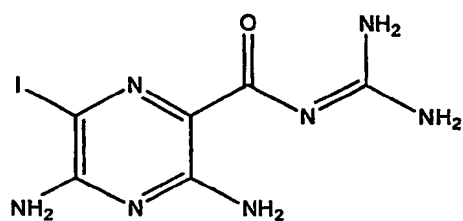
5-(N,N-Dimethyl)amiloride hydrochloride comprising the structure



5-tert-butylamino-amiloride comprising the structure

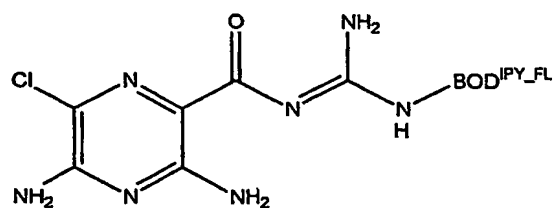


6-Iodoamiloride comprising the structure

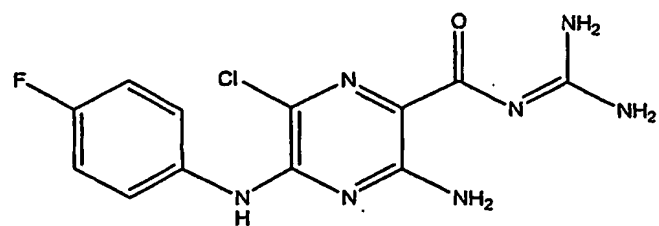


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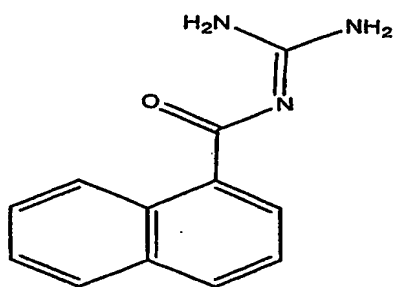
Bodipy-FL Amiloride comprising the structure



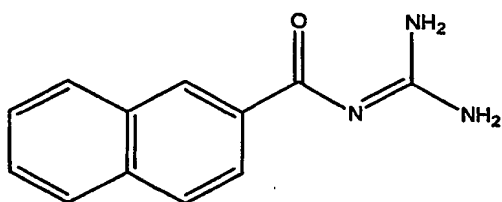
5-(4-fluorophenyl)amiloride comprising the structure



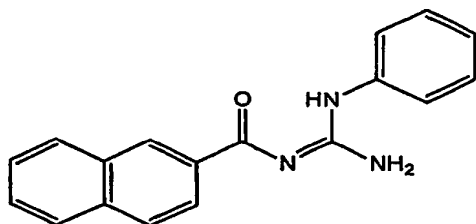
1-naphthoylguanidine comprising the structure



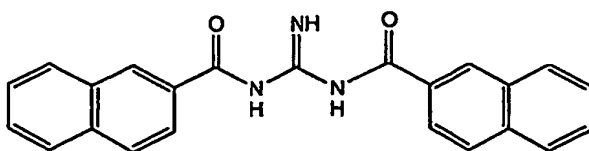
2-naphthoylguanidine comprising the structure



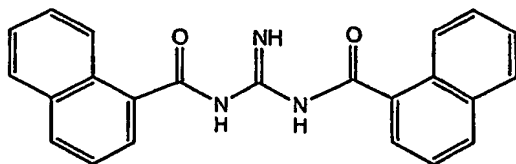
5 N-(2-naphthoyl)-N'-phenylguanidine comprising the structure



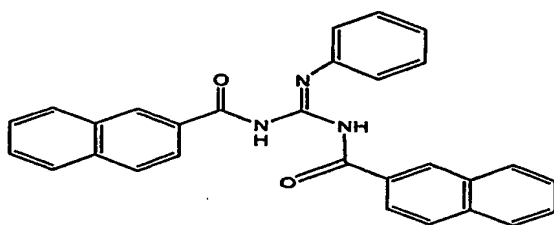
N,N'-bis(2-naphthoyl)guanidine comprising the structure



N,N'-bis(1-naphthoyl)guanidine comprising the structure

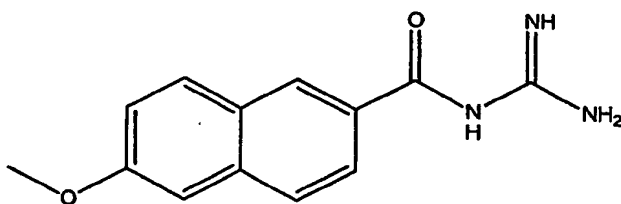


N,N'-bis(2-naphthoyl)-N''-phenylguanidine comprising the structure

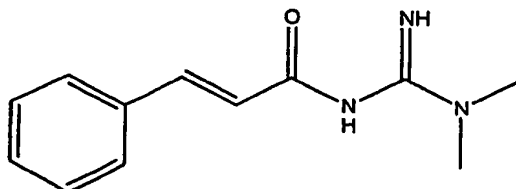


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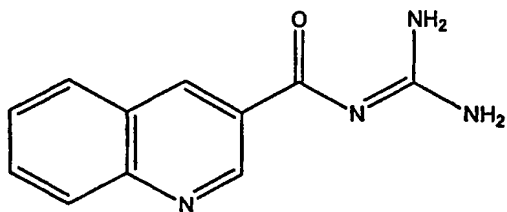
6-methoxy-2-naphthoylguanidine comprising the structure



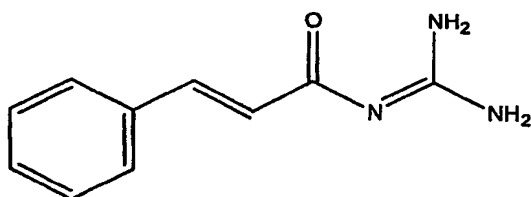
N-Cinnamoyl-N',N'-dimethylguanidine comprising the structure



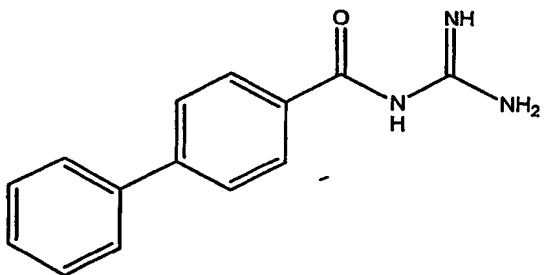
3-quinolinoylguanidine comprising the structure



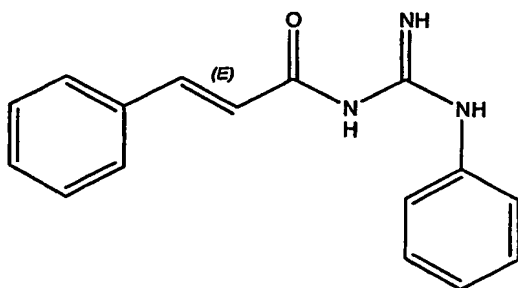
cinnamoylguanidine comprising the structure



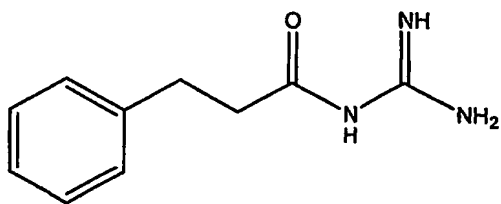
5 4-phenylbenzoylguanidine comprising the structure



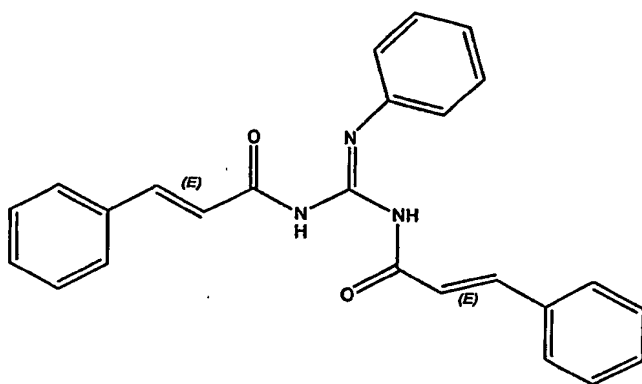
N-(cinnamoyl)-N'-phenylguanidine comprising the structure



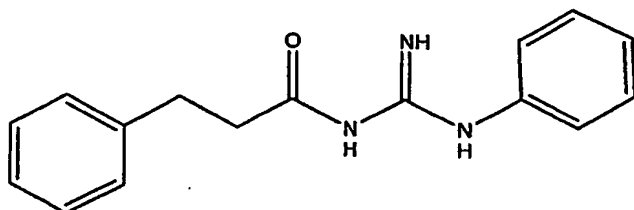
(3-phenylpropanoyl)guanidine comprising the structure



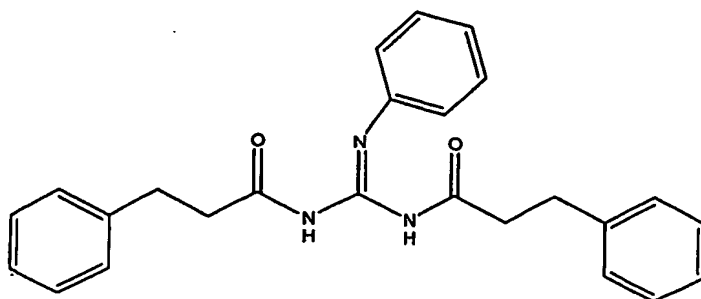
N,N'-bis-(cinnamoyl)-N''-phenylguanidine comprising the structure



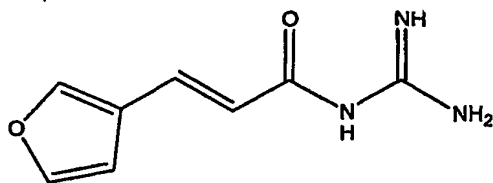
N-(3-phenylpropanoyl)-N'-phenylguanidine comprising the structure



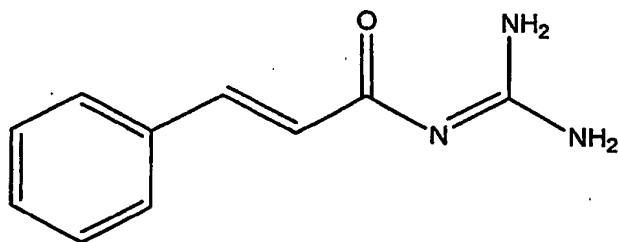
5 N,N'-bis(3phenylpropanoyl)-N''-phenylguanidine comprising the structure



trans-3-furanacryoylguanidine comprising the structure



One of the preferred compounds of the invention comprises the structure



5.

cinnamoylguanidine (Bit036).

### BRIEF DESCRIPTIONS OF THE DRAWINGS

**Figure 1.** SARS E protein ion channel activity observed in NaCl solutions after exposure of lipid bilayer to 3-10 $\mu$ g of E protein. **A.** The closed state is shown as solid line, openings are derivations from the line. Scale bar is 300ms and 5pA. The CIS chamber contained 50mM NaCl in 5mM HEPES buffer pH 7.2, the TRANS chamber contained 500mM NaCl in 5mM HEPES buffer pH 7.2. The CIS chamber was earthed and the TRANS chamber was held at various potentials between -100 to +100mV. **B.** Largest single opening events of a single channel.

**Figure 2.** Bit036 inhibits SARS E protein ion channel activity in NaCl solution. **A.** Representative currents at holding potential of -40mV. Scale bar is 300ms and 5pA. E protein ion channel activity and E protein channel activity after the addition of 100 $\mu$ M Bit036. **B.** All points histogram at holding potential of -40mV. E protein ion channel activity before and after the addition of 100 $\mu$ M Bit036. **C.** Average current (pA), before formation of E protein ion channel, E protein ion channel activity and after addition of 100 $\mu$ M Bit036.

**Figure 3.** SARS E protein ion channel activity observed after exposure of lipid bilayer to 3-10 $\mu$ g of E protein **A.** The closed state is shown as solid line, openings are derivations from the line. Scale bar is 150mS and 5pA. The CIS chamber contained 50mM KCl in 5mM HEPES buffer pH 7.2, the TRANS chamber contained 500mM KCl in 5mM HEPES buffer pH 7.2 The CIS chamber was earthed and the TRANS chamber was held at various potentials between -100 to +100mV. **B.** Largest single opening events of a single channel.

**Figure 4.** Bit036 inhibits SARS E protein ion channel activity in KCl solutions **A.** Representative currents at holding potential of -40mV. Scale bar is 100mS and 3pA. E protein ion channel activity and E protein ion channel activity after the addition of 100 $\mu$ M Bit036. **B.** Average current (pA), before formation of E protein ion channel, E protein ion channel activity and after the addition of 100 $\mu$ M Bit036.

**Figure 5.** SARS E protein ion channel activity observed after exposure of lipid bilayer to 3-10 $\mu$ g of E protein N-terminal domain in KCl solution **A.** The closed state is shown as solid line, openings are deviations from the line. Scale bar is 100mS and 5pA. The CIS chamber contained 50mM KCl in 5mM HEPES buffer pH 7.2, the TRANS chamber contained 500mM KCl in 5mM HEPES buffer pH 7.2 The CIS chamber was earthed and the TRANS chamber was held at various potentials between -100 to +100mV. **B.** Largest single opening events of a single channel.

## DETAILED DESCRIPTION OF THE INVENTION

With relevance to coronaviruses other than the SARS virus, and without being bound by any particular theory or mechanism of action, the effect of the compounds of the present invention appears to be exerted via the inhibition of, or other negative effect on, the E protein of the SARS virus which was shown in the present studies to act as an ion channel. As similar E proteins are present in other coronaviruses, the compositions and methods of the present invention would have utility in the inhibition and/or treatment of infections by all coronaviruses.

The table below provides examples of coronaviruses which could be inhibited or infection treated by the compositions and methods of the present invention.

**Table 1 Known coronaviruse isolates**

<b>Group 1 species</b>
<u>Canine coronavirus</u>
<u>Canine enteric coronavirus (strain INSAVC-1)</u>
<u>Canine enteric coronavirus (strain K378)</u>
<u>Feline coronavirus</u>
<u>Feline enteric coronavirus (strain 79-1683)</u>
<u>Feline infectious peritonitis virus (FIPV)</u>
<u>Human coronavirus 229E</u>
<u>Porcine epidemic diarrhea virus</u>
<u>Porcine epidemic diarrhea virus (strain Br1/87)</u>
<u>Porcine epidemic diarrhea virus (strain CV777)</u>
<u>Transmissible gastroenteritis virus</u>
<u>Porcine respiratory coronavirus</u>
<u>Porcine transmissible gastroenteritis coronavirus (STRAIN FS772/70)</u>
<u>Porcine transmissible gastroenteritis coronavirus (strain Miller)</u>
<u>Porcine transmissible gastroenteritis coronavirus (strain Neb72-RT)</u>
<u>Porcine transmissible gastroenteritis coronavirus (STRAIN PURDUE)</u>
<b>Group 2 species</b>
<u>Bovine coronavirus</u>
<u>Bovine coronavirus (STRAIN F15)</u>
<u>Bovine coronavirus (strain G95)</u>
<u>Bovine coronavirus (STRAIN L9)</u>
<u>Bovine coronavirus (strain LSU-94LSS-051)</u>
<u>Bovine coronavirus (STRAIN LY-138)</u>
<u>Bovine coronavirus (STRAIN MEBUS)</u>
<u>Bovine coronavirus (strain OK-0514-3)</u>
<u>Bovine coronavirus (strain Ontario)</u>
<u>Bovine coronavirus (STRAIN QUEBEC)</u>
<u>Bovine coronavirus (STRAIN VACCINE)</u>
<u>Bovine enteric coronavirus (strain 98TXSF-110-ENT)</u>
<u>Canine respiratory coronavirus</u>
<u>Chicken enteric coronavirus</u>
<u>Human coronavirus OC43</u>
<u>Murine hepatitis virus</u>
<u>Murine coronavirus (strain DVIM)</u>
<u>Murine hepatitis virus (strain A59)</u>
<u>Murine hepatitis virus (strain JHM)</u>
<u>Murine hepatitis virus (strain S)</u>
<u>Murine hepatitis virus strain 1</u>
<u>Murine hepatitis virus strain 2</u>
<u>Murine hepatitis virus strain 3</u>
<u>Murine hepatitis virus strain 4</u>
<u>Murine hepatitis virus strain ML-11</u>

<u>Porcine hemagglutinating encephalomyelitis virus</u>
<u>Porcine hemagglutinating encephalomyelitis virus (strain 67N)</u>
<u>Porcine hemagglutinating encephalomyelitis virus (strain IAF-404)</u>
<u>Puffinosis virus</u>
<u>Rat coronavirus</u>
<u>Rat coronavirus (strain 681)</u>
<u>Rat coronavirus (strain NJ)</u>
<u>Rat sialodacryoadenitis coronavirus</u>
<b><u>Group 3 species</u></b>
<u>Turkey coronavirus</u>
<u>Turkey coronavirus (strain Indiana)</u>
<u>Turkey coronavirus (strain Minnesota)</u>
<u>Turkey coronavirus (strain NC95)</u>
<u>Avian infectious bronchitis virus</u>
<u>Avian infectious bronchitis virus (STRAIN 6/82)</u>
<u>Avian infectious bronchitis virus (strain Arkansas 99)</u>
<u>Avian infectious bronchitis virus (strain Beaudette CK)</u>
<u>Avian infectious bronchitis virus (strain Beaudette M42)</u>
<u>Avian infectious bronchitis virus (strain Beaudette US)</u>
<u>Avian infectious bronchitis virus (strain Beaudette)</u>
<u>Avian infectious bronchitis virus (strain D1466)</u>
<u>Avian infectious bronchitis virus (strain D274)</u>
<u>Avian infectious bronchitis virus (strain D3896)</u>
<u>Avian infectious bronchitis virus (strain D41)</u>
<u>Avian infectious bronchitis virus (strain DE072)</u>
<u>Avian infectious bronchitis virus (strain GRAY)</u>
<u>Avian infectious bronchitis virus (strain H120)</u>
<u>Avian infectious bronchitis virus (strain H52)</u>
<u>Avian infectious bronchitis virus (strain KB8523)</u>
<u>Avian infectious bronchitis virus (strain M41)</u>
<u>Avian infectious bronchitis virus (strain PORTUGAL/322/82)</u>
<u>Avian infectious bronchitis virus (strain SAIB20)</u>
<u>Avian infectious bronchitis virus (strain UK/123/82)</u>
<u>Avian infectious bronchitis virus (strain UK/142/86)</u>
<u>Avian infectious bronchitis virus (strain UK/167/84)</u>
<u>Avian infectious bronchitis virus (strain UK/183/66)</u>
<u>Avian infectious bronchitis virus (strain UK/68/84)</u>
<u>Avian infectious bronchitis virus (strain V18/91)</u>
<u>Avian infectious bronchitis virus (strain Vic S)</u>
<u>Avian infectious laryngotracheitis virus</u>
<b><u>Preliminary Group 4 species</u></b>
<u>SARS coronavirus</u>
<u>SARS coronavirus Beijing ZY-2003</u>
<u>SARS coronavirus BJ01</u>
<u>SARS coronavirus BJ02</u>
<u>SARS coronavirus BJ03</u>
<u>SARS coronavirus BJ04</u>
<u>SARS coronavirus CUHK-Su10</u>

<u>SARS coronavirus CUHK-W1</u>
<u>SARS coronavirus Frankfurt 1</u>
<u>SARS coronavirus GZ01</u>
<u>SARS coronavirus HKU-39849</u>
<u>SARS coronavirus Hong Kong ZY-2003</u>
<u>SARS coronavirus Hong Kong/03/2003</u>
<u>SARS coronavirus HSR 1</u>
<u>SARS coronavirus Sin2500</u>
<u>SARS coronavirus Sin2677</u>
<u>SARS coronavirus Sin2679</u>
<u>SARS coronavirus Sin2748</u>
<u>SARS coronavirus Sin2774</u>
<u>SARS coronavirus Taiwan</u>
<u>SARS coronavirus Taiwan JC-2003</u>
<u>SARS coronavirus Taiwan TC1</u>
<u>SARS coronavirus Taiwan TC2</u>
<u>SARS coronavirus Tor2</u>
<u>SARS coronavirus TW1</u>
<u>SARS coronavirus TWC</u>
<u>SARS coronavirus Urbani</u>
<u>SARS coronavirus Vietnam</u>
<u>SARS coronavirus ZJ-HZ01</u>
<u>SARS coronavirus ZJ01</u>
<u>unclassified coronaviruses</u>
<u>Bovine respiratory coronavirus (strain 98TXSF-110-LUN)</u>
<u>Human enteric coronavirus 4408</u>
<u>Enteric coronavirus</u>
<u>Equine coronavirus</u>
<u>Equine coronavirus NC99</u>

The present invention will now be described in more detail with reference to specific but non-limiting examples involving the use of cinnamoylguanidine (Bit036) and the SARS virus. It will be clear from the description herein that other coronaviruses and other compounds may be used effectively in the context of the present invention.

## EXAMPLES

### *Example 1: Materials and experimental protocols*

A peptide corresponding to the full-length SARS-CoV (isolate Tor2 and Urbani) E protein (MYSFVSEETGTLIVNSVLLFLAFVFLVTLAILTALRLCA YCCNIVNVSLVKPTVYVYSRVKNLNSSEGVPDLLV) and a second peptide comprising the first 40 amino acids of the full length E protein which correspond to the transmembrane domain (MYSFVSEETGTLIVNSVLLFLAFVVF

LLVTLAILTALRLC) were synthesized manually using Fmoc chemistry and solid phase peptide synthesis. The synthesis was done at the Biomolecular Resource Facility (John Curtin School of Medical Research, ANU, Australia) using a Symphony<sup>R</sup> Peptide Synthesiser from Protein Technologies Inc. (Tucson, AZ, USA) according to the manufacturers instructions. The SARS virus E protein was resuspended at 1mg/ml in 2,2,2-trifluoroethanol. The SARS virus E protein's ability to form ion channels was tested on a Warner (Warner instruments, Inc. 1125 Dixwell Avenue, Hamden, CT 06514) bilayer rig as follows; A lipid mix of 3:1:1, 1-Palmitoyl-2-oleoyl phosphatidyl Ethanolamine: 1-Palmitoyl-2-oleoyl phosphatidyl Serine: 1-Palmitoyl-2-oleoyl phosphatidyl choline in CHCl<sub>3</sub> was dried under N<sub>2</sub> gas and resuspended to 50mg/ml in n-decane. Bilayers were painted across a circular hole of approximately 100µm diameter in a Warner plastic cup separating aqueous solution in the CIS and TRANS chambers. The CIS chamber contained a solution of 500mM NaCl or KCl, in a 5mM HEPES buffer pH 7.2, the TRANS chamber contained a solution of 50mM NaCl or KCl, in a 5mM HEPES buffer pH 7.2. Silver electrodes coated in chloride with 2% agarose bridges are placed in the CIS and TRANS chamber solutions. The CIS chamber is earthed and the TRANS chamber is amplified. Voltage up to +/-200mV can be applied to the TRANS chamber. SARS virus E protein (3-10µg) was added to the CIS chamber stirring until channel activity was detected. The TRANS chamber was held at various holding potentials ranging between +100 to -100mV and currents were amplified using a Warner model BD-525D amplifier with sampling 5kHz and filtered at 1kHz before being digitally recorded directly using the Data Collect software developed by Mr Bernie Keys (BioResearch Electronics, Canberra, Australia)

For experiments testing the ability of compounds to inhibit E protein ion channel activity, once channel activity was observed 100µM to 200µM of compound was added to the CIS chamber while stirring for 30 seconds. Currents were recorded before channel activity, during channel activity and after the addition of the drug. For the purpose of this experiment the drug was cinnamoylguanidine (Bit036), a compound which was shown in earlier

*experiments to be antiviral and to inhibit ion channel proteins from other viruses.*

## **Results and Discussion.**

The present investigations have shown that SARS virus E protein forms ion channels on planar lipid bilayers. E protein ion channel activity was observed in over 60 independent experiments. The SARS virus E protein ion channel with 50mM NaCl in the CIS and 500mM NaCl in the TRANS chambers reverses at between +30 to +40mV, indicating that the E protein ion channel is selective for sodium over chloride ions (Figure 1 A and B). This is the first demonstration that E proteins from coronaviruses form ion channels on planar lipid bilayers. The SARS virus E protein also forms ion channels in the presence of 50mM KCl in the CIS and 500mM NaCl in the TRANS. SARS virus E protein in KCl reversed at between +20 and +30mV, indicating that the E protein ion channel is selective for potassium over chloride ions, but not as selective for potassium as it is for sodium ions (Figure 2A and 2B). The first 40 amino acids of the N-terminal which contains the hydrophobic domain of the SARS virus E protein is sufficient for the formation of ion channels on planar lipid bilayers. The N-terminal domain in the presence of 50mM KCl in the CIS and 500mM NaCl in the TRANS reversed at between +20 and +30mV, indicating that the N-terminal E protein ion channel has the same selectivity as the full-length E protein ion channel.

The SARS virus E protein ion channel activity on planar lipid bilayers in NaCl and KCl solutions inhibited by addition of between 100 $\mu$ M to 200 $\mu$ M Bit036 to the CIS chamber (Figure 2 and Figure 4). Inhibition or partial inhibition of the E protein ion channel activity by Bit036 has been observed in seven independent experiments in NaCl solution and four independent experiments in KCl solution.

All known coronaviruses encode an E protein with a hydrophobic N-terminus transmembrane domain therefore all coronaviruses E proteins could form ion channels on planar lipid bilayers. This indicates that the E protein could be a suitable target for antiviral drugs and potentially stop the spread of coronavirus from infected host cells. Drugs that block the E protein ion channel could be effective antiviral therapy for the treatment of several significant

human and veterinary coronavirus diseases including SARS and the common cold.

*Example 2. Plaque assay screen of anti-coronavirus compounds*

Compounds can be screened for their ability to inhibit budding of various different coronaviruses by a number of well known assays, including the plaque assay. Suitable methods are described in standard texts, such as for example "Fundamental techniques in virology", ed. / by K. Habel and N.P. Salzman. N.Y., Academic Press, 1969 and Basic medical virology. / Balt., Williams & Wilkins, 1966 or Adolph, K.W. (ed) (1994) Molecular virology techniques". In: Methods in Molecular Genetics, Vol. 4. New York: Academic Press, both of which are incorporated in their entirety herein by reference.

Briefly the procedure is as follows.

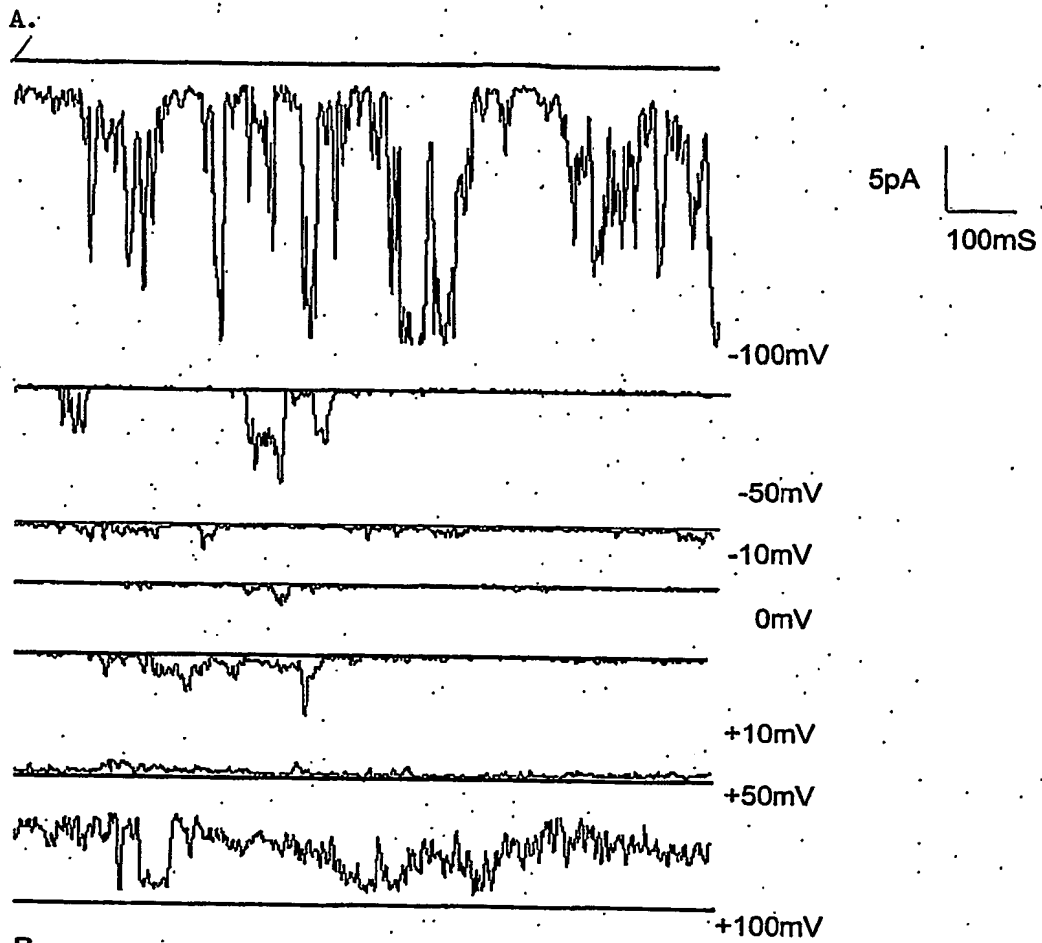
Cells susceptible to infection with the coronavirus to be tested (for example MRC-5 cells for human coronavirus 229E or OC 43) are plated into 6 well plates and grown to confluence. Once cells reach confluence the culture supernatant are removed and the cells infected with the coronavirus to be tested at a multiplicity of infection (MOI) 5. After 1 hour of infection at between 33-37°C, the virus is removed and the cells washed in culture media and replaced with 1ml of culture media or 1ml 1% agarose in culture media overlay. Drug to be tested is added at various concentrations to the separate wells of coronavirus infected cells. The coronavirus infected cells are incubated at between 33-37°C for 4-12 days or until plaques are present.

The culture supernatant are removed and the cells stained with neutral red or crystal violet stain or the agarose is stained with dye. Plaques are counted and the plaque forming units (PFU) calculated. PFU are compared between wells that had drug added against wells without drug. If the drug inhibits the coronavirus then there is a reduction of plaques present for that well.

Although the invention has been described with reference to certain examples and preferred embodiments, it will be understood by those skilled in the art that variations and modifications in keeping with the principles and spirit of the invention described herein are also encompassed.

DATED this 25th Day of July 2003  
BALDWIN SHELSTON WATERS  
Attorneys for: Biotron Limited

Figure 1.



B.

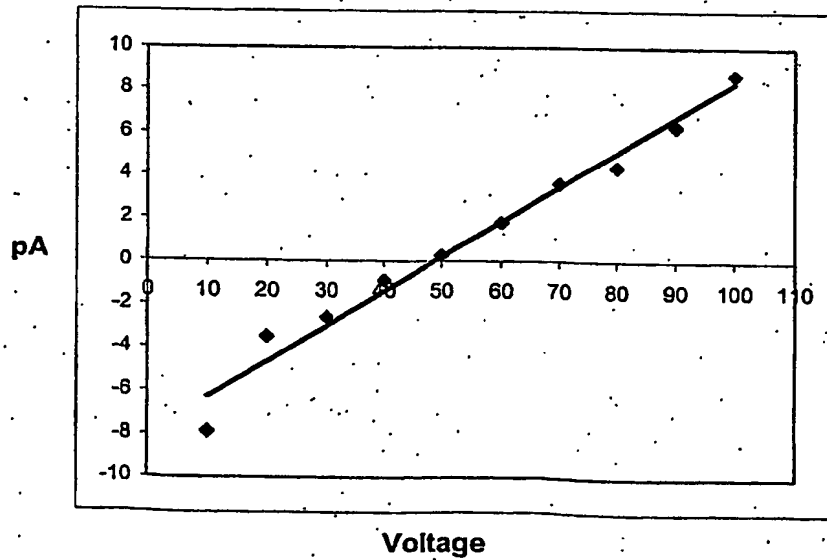
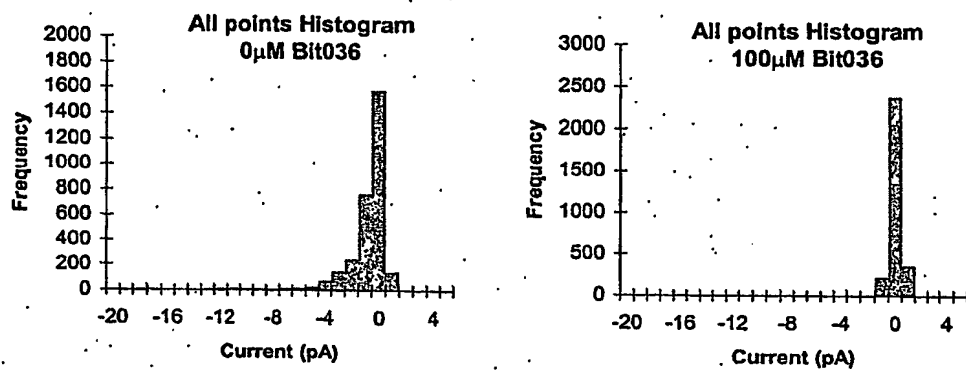


Figure 2. A.



B.



C.

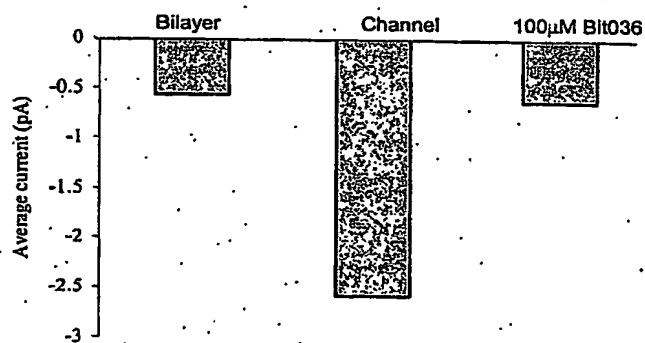
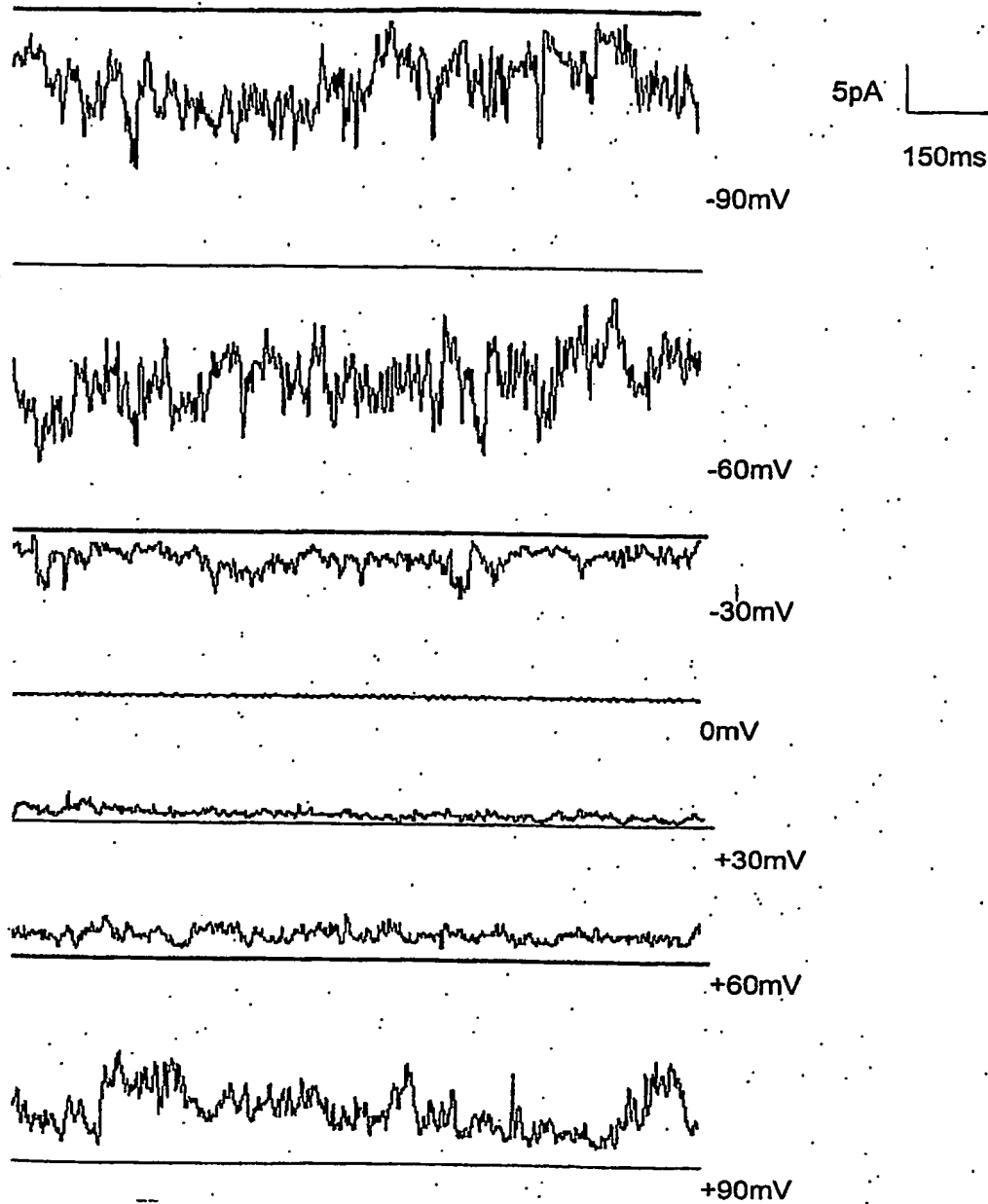


Figure 3A.



B.

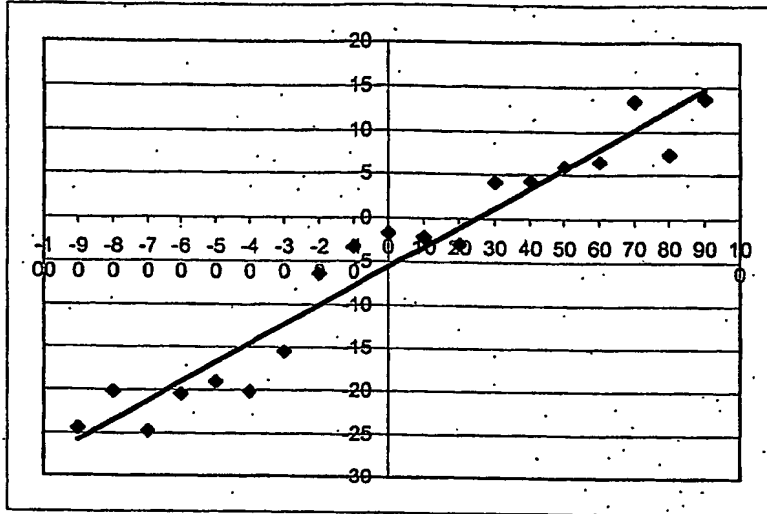
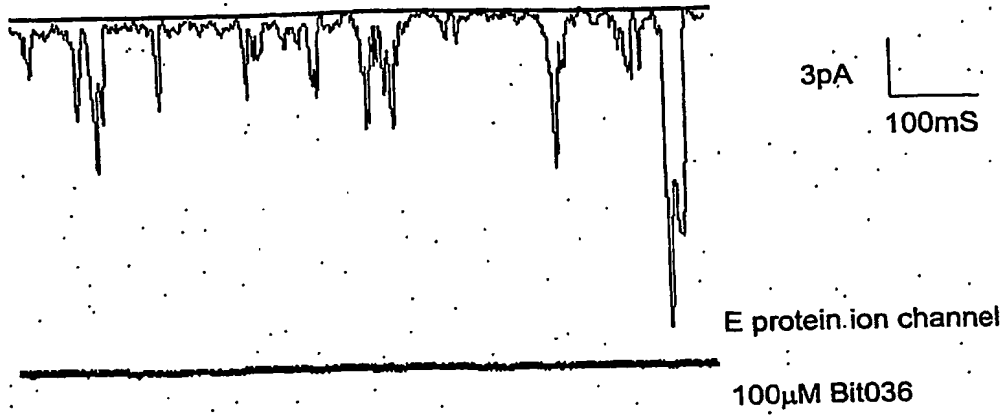


Figure 4. A.



B.

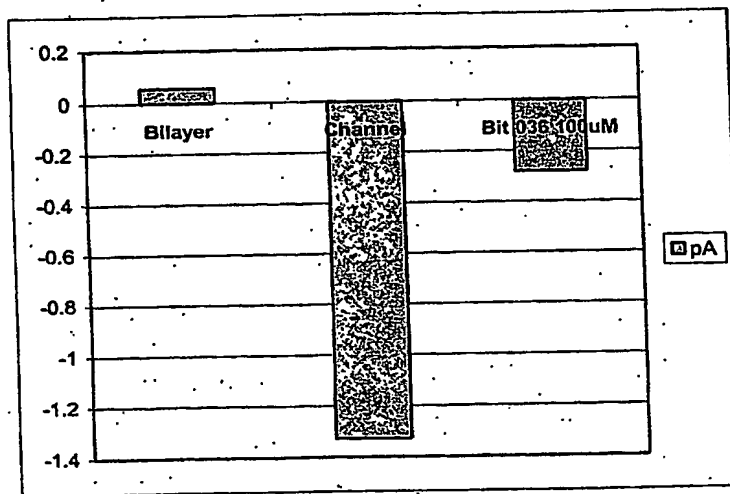
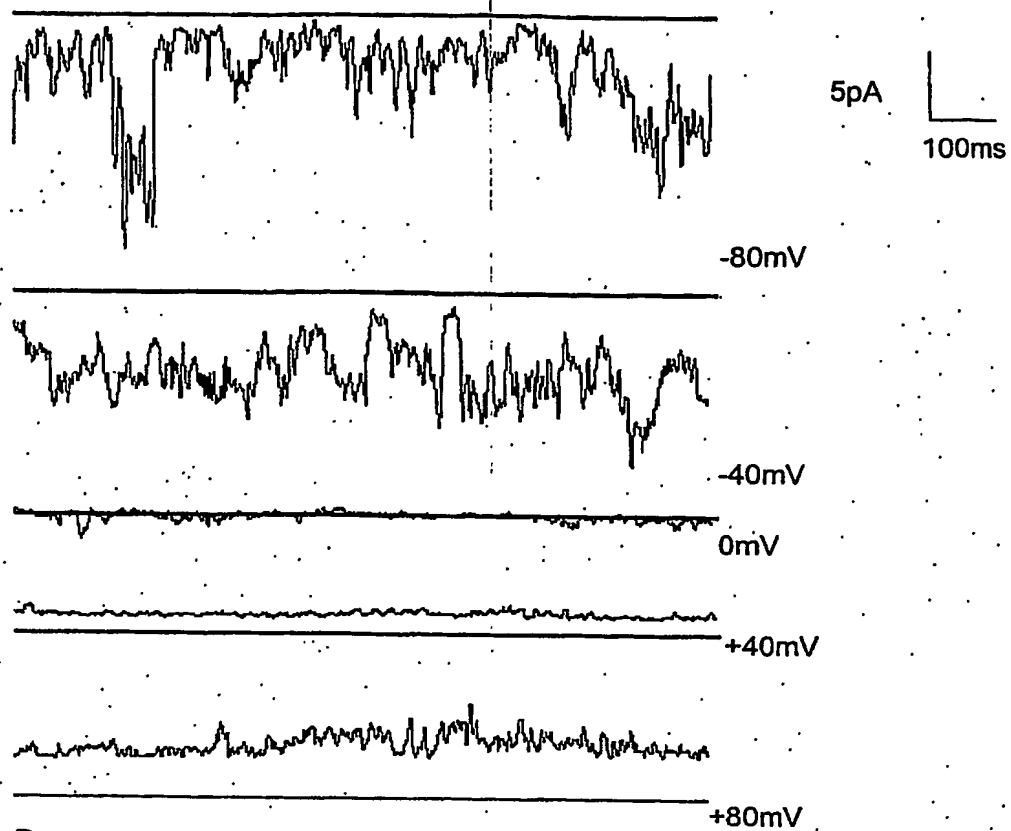
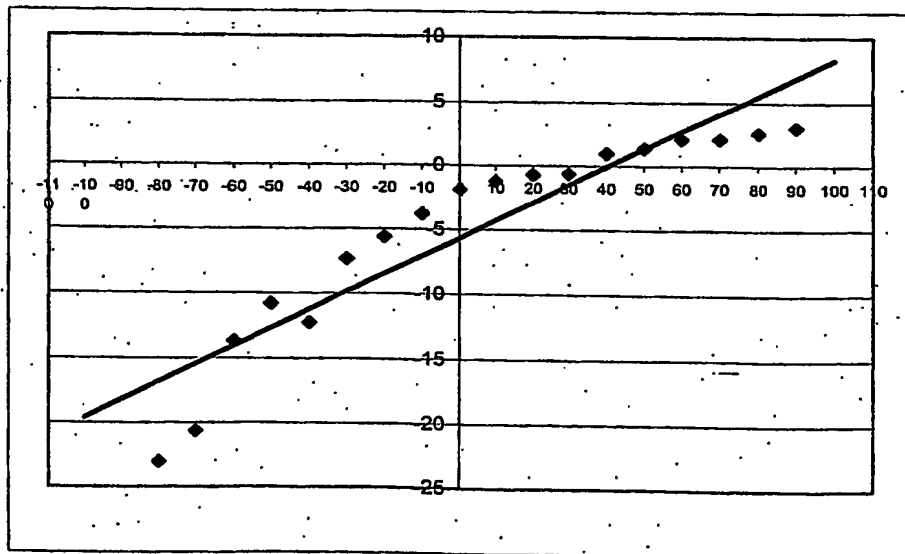


Figure 5.



B.

pA



Voltage

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